

A novel NINJ1-mediated regulatory step is essential for active membrane rupture and common to different cell death pathways

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EVALUATION OF



NINJ1 mediates plasma membrane rupture during lytic cell death

Kayagaki *et al.*

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Plasma membrane rupture (PMR), the final event in lytic cell death that is in part responsible for the release of pro-inflammatory signals, was believed to be a passive event that followed osmotic swelling. Kayagaki *et al.*¹ have discovered that PMR is, in fact, mediated by ninjurin-1 (NINJ1), adding a novel regulatory step that is conserved across different types of lytic cell death, such as pyroptosis, necroptosis, and apoptosis. PMR is dependent on NINJ1 oligomerization, which is mediated by its highly conserved putative N-terminal α -helix. *In vivo* data suggest that the NINJ1-dependent secretome that is released upon PMR is likely to modulate antimicrobial host defense, suggesting this additional regulatory step also has physiological relevance.

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Background

Programmed cell death (PCD) pathways play a central role in eliminating cells that are either at the end of their lifespan or pose a potential threat to a multicellular organism or a population of unicellular cells. A key evolutionary driving force of PCD pathways is its use as a last resort mechanism to prevent uncontrolled cell growth or to limit infection. Apoptosis, which represents a “silent” type of cell death, was the first PCD pathway to be characterized. While the cell is disintegrated in a coordinated fashion, its membrane integrity is maintained such that no intracellular components leak out^{2,3}. This is important because certain cytosolic components can act as damage-associated molecular patterns (DAMPs) that can trigger potent inflammatory responses⁴. Further, specific signals are generated during apoptosis to ensure that dead cells are removed in an immunologically-silent, non-inflammatory manner by a phagocytic process known as efferocytosis.

In addition, lytic types of cell death have been characterized as those that result in the complete disintegration of the cell. Unlike apoptosis, these lytic types of cell death are highly pro-inflammatory, which is attributed to the release of cytosolic content. Necrosis is used as an umbrella term for these types of cell death to distinguish them from apoptosis. While necrosis can occur in an accidental, uncontrolled fashion (e.g., traumatic cell injury) or triggered by exogenous molecules (e.g., pore-forming toxins), it has also been appreciated that necrosis can be executed in a programmed manner⁵. Here, two types of PCD pathways have been well-characterized: Pyroptosis is governed by the caspase-dependent cleavage of gasdermins, which upon cleavage form large pore structures within the plasma membrane⁶. While initially identified downstream of inflammatory caspases that are activated by inflammasomes, pyroptosis can

also occur as a late event in apoptosis (here often referred to as “secondary necrosis”)⁷. Necroptosis, on the other hand, is governed by the activation of the pseudokinase mixed lineage kinase domain-like protein (MLKL)³. Analogous to gasdermins, activated MLKL is recruited to the plasma membrane, forming a channel or pore-like structure^{3,8}. Both these events result in the dissipation of ion gradients and the unabated influx of water. This, in turn, results in the osmotic swelling of the cell, which is followed by plasma membrane rupture (PMR). The prevailing view was that the ensuing PMR is a passive event caused by insurmountable mechanical stress resulting from cell swelling *per se*.

Main contributions and importance

The conclusions drawn by Kayagaki and colleagues¹ challenge the canonical view regarding PMR during cell death. Rather than being a passive and uncontrolled event, PMR might be actively regulated, exhibiting greater complexity than once expected. The authors discovered that, following cell death stimuli, the adhesion protein *ninjurin-1* (NINJ1) accumulates at the cell surface and oligomerizes — a critical event for PMR (Figure 1B). This event occurs downstream of the gasdermin-mediated pore formation; therefore, NINJ1-mediated PMR is an additional regulatory step within cell death pathways. While gasdermin pores alone result in osmotic swelling, the mechanical stress generated is not sufficient to drive PMR. The authors conclude that this NINJ1-mediated step occurs not only in pyroptosis but also in necrosis (downstream of bacterial pore-forming toxins and consequent swelling) and post-apoptosis (downstream of ATP depletion and resultant swelling).

Of note, the authors discovered that PMR is not essential for cell death. NINJ1-deficient bone marrow-derived macrophages well and develop

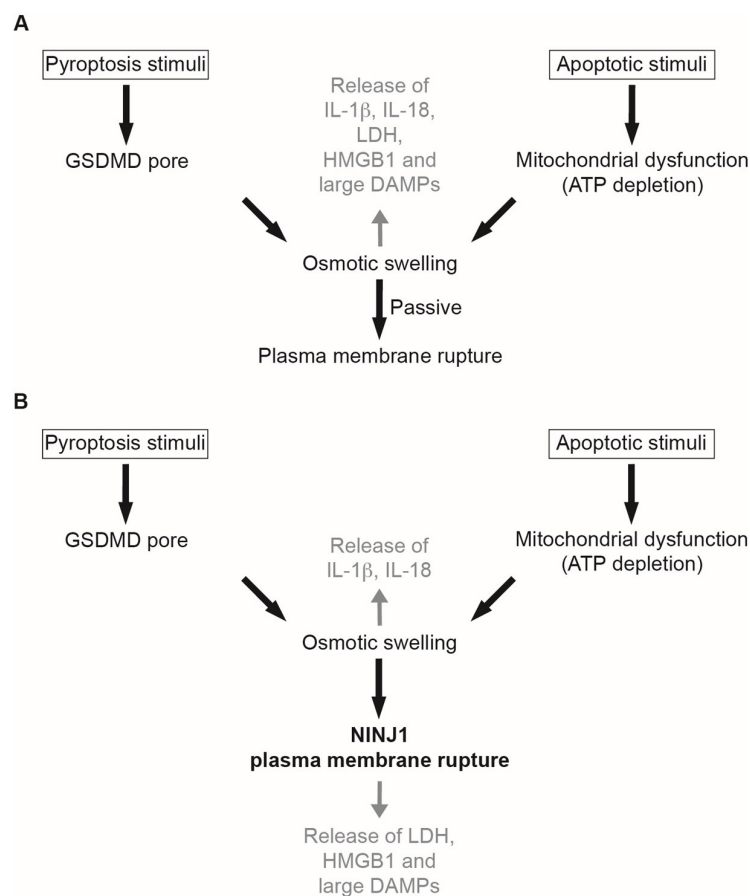


Figure 1. Long-held (A) and proposed (B) models for pyroptosis- and post-apoptosis-related plasma membrane rupture (PMR)

Prior to Kayagaki *et al.*¹, PMR in pyroptosis and post-apoptosis were considered passive events caused by the mechanical stress induced by osmotic swelling (A). In the proposed model (B), osmotic swelling does not drive PMR. Instead, ninjurin-1 (NINJ1)-mediated pores are formed that increases plasma membrane permeability, causing a rupture that not only releases cytosolic content, including lactate dehydrogenase (LDH), but also large danger-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1). Previously, these were thought to be co-released with pro-inflammatory cytokines. NINJ1, therefore, is a mediator of PMR and has pro-inflammatory roles.

bubble-like herniations that do not disintegrate (in contrast to their wild-type counterparts), yet they present features of cell death (including loss of ATP, mitochondrial membrane potential, and motility). However, NINJ1-dependent PMR plays a critical inflammatory role by promoting the release of pro-inflammatory DAMPs, such as HMGB1. Quantitatively, ~780 molecules/cells were found to be secreted in a NINJ1-dependent manner in macrophages stimulated with nigericin or cytoplasmic lipopolysaccharide (LPS) (inducers of pyroptosis). Therefore, the secretome associated with NINJ1-dependent PMR may be

important for host defense against bacteria. Consistent with this, *Ninj1*^{-/-} mice showed increased susceptibility to infection with *Citrobacter rodentium*.

Furthermore, the mechanistic insight gained separates the upstream gasdermin-mediated events from later NINJ1-mediated events within the cell lysis cascade. With this knowledge at hand, the release of cytosolic content and larger DAMPs can now be studied independently of gasdermin pore formation and the consequent release of pro-inflammatory cytokines. In NINJ1-deficient macrophages, cytokine

release (e.g., IL-1 β) was achieved through the gasdermin pore and, therefore, unaffected, while the release of large cytosolic content (including lactate dehydrogenase and other large molecules that can act as potential DAMPs) was significantly inhibited within the time-frame assessed. Thus, NINJ1-mediated PMR allows the release of larger molecules than gasdermin-mediated pores.

Mechanistically, Kayagaki *et al.* found that the release of cytosolic content and large DAMPs was dependent on NINJ1 oligomerization for the formation of a putative pore. Mutations that negatively affected oligomerization (namely *K45Q* and *A59P*) impaired NINJ1-mediated PMR. These mutations are in an N-terminal domain that is believed to form an extracellular α -helix that is highly conserved across NINJ isoforms capable of cytotoxicity.

Taken together, this is the first account of cell lytic and pro-inflammatory roles for NINJ1.

Open questions

The identification of a common regulatory mechanism within cell death pathways raises several questions. Firstly, what is the driver of NINJ1 oligomerization, and how does it occur specifically under certain types of cell death? While the authors acknowledge the lack of structural and biophysical analyses into NINJ1 oligomerization, they propose that an increase in cell volume may trigger activation of NINJ1, through intrinsic lipid/curvature-sensing properties. This should be further investigated, particularly because key residues for NINJ1 oligomerization and toxicity that lie in the putative extracellular α -helix have been previously associated with other cytotoxic, pore-forming proteins, such as α -synuclein, and found to influence membrane-binding properties⁹. Secondly, why is NINJ1-mediated PMR only partially

required for necroptosis, while it is indispensable for pyroptosis, necrosis triggered by pore-forming toxins, and late apoptosis? Investigating these different types of cell death pathways at a mechanistic level could help to explain whether NINJ1 activation is driven by its biophysical properties since necroptotic cells also undergo swelling but do not require NINJ1-mediated PMR. Thirdly, since the authors conclude that oligomerization mediates PMR and the release of pro-inflammatory DAMPs, is the role of NINJ1 impaired if oligomerization is inhibited? A recent pre-print has shown that glycine treatment phenocopies NINJ1 deficiency by inhibiting oligomerization¹⁰, although mechanistic details are lacking. Therefore, investigating the effect of glycine treatment on the NINJ1-dependent secretome would contribute to assessing the correlation between NINJ1 oligomerization, its pro-inflammatory functions, and its role in the loss of membrane integrity during cell death.

Going forward, the role of NINJ1 in human cells should also be assessed. What is the effect of NINJ1 deficiency on human cells and tissue homeostasis? How does the interplay/co-existence between cytotoxic and non-cytotoxic ninjurin isoforms dictate cell fate? Further, what is the primordial function of ninjurin through evolution (i.e., how has it evolved to become a cell lysis molecule, considering its established cell-cell adhesion functions)? Answering these questions would elucidate any species-specific functions and help us to understand whether the role of NINJ1 across cell death pathways is predominantly a pro-inflammatory and/or lytic one.


It will also be interesting to further explore whether NINJ1 is a potential therapeutic target — an investigation has been initiated by the authors using a monoclonal NINJ1 antibody *in vitro*. Finally, the identification of other PMR mediators could also be beneficial for the field.

Conclusion

Kayagaki *et al.* have identified NINJ1 as a common mediator of PMR in lytic types of programmed cell death that have pro-inflammatory roles. Importantly, this has challenged the long-held hypothesis that cell death-related PMR is a

passive event. Furthermore, the existence of a novel regulatory step highlights further molecular complexity within cell death mechanisms, which was not anticipated. Therefore, this pioneering work, arching from genetic discovery and biochemical workup to *in vivo* relevance, will certainly influence future research.

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